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Endophyte-produced alkaloids may negatively affect herbivores. They may also cascade up the food web and affect the third trophic level, predators and parasites of herbivores. In this study, I examine the effect that endophyte infection and alkaloids have on two different strains of bird cherry-oat aphids (*Rhopalosiphum padi*) and their natural enemy, the ladybird beetle (*Coleomegilla maculata*). The effect of alkaloid levels produced by the endophyte-infected (E+) versus uninfected (E-) sleepygrass plants (*Achnatherum sibiricum*) was examined on aphid abundances. The effect of hybrid, non-hybrid, and uninfected *Neotyphodium* sp. endophytes hosted by Arizona fescue (*Festuca arizonica*) was examined on the abundance and feeding preference of aphids, the feeding preference of ladybird beetles, and on first and second-generation ladybird beetle development. Contrary to our expectations, aphid strains did not differ in their response to endophyte infection. However, there was a trend for lower aphid numbers on hybrid Arizona fescue and aphid preference for endophyte-free plants. Endophyte infection had large effects on second-generation ladybird beetles. Ladybird beetles on endophyte-infected plants had lower adult survival and weight than all other infection categories. My experiments demonstrate that variation in endophyte genotype and hence, alkaloids can negatively affect herbivores, but may also have negative effects on natural enemies, which may nullify their benefits to the plant.

EFFECTS OF *NEOTYPHODIUM* ENDOPHYTES ON MULTITROPHIC INTERACTIONS

by

Melissa Elizabeth Robbins

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the Faculty of The Graduate School at
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Approved by

_____

Committee Chair

This thesis is dedicated to God and my mother, who have shown me how to learn and have been my support system through these twenty years of schooling. This is for all of your love. It is also dedicated to my beautiful sister Elisha, whom we miss dearly. Your life will always be celebrated and you are my angel. This Master's degree is for all three of you! Thank you.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Epichloid Endophytes in Native North American Grass Species

The term endophyte refers generally to any microorganism that lives inside of a plant without causing disease (Clay and Schardl 2002). Like the term "epiphyte" that refers to organisms living on a plant's surface, the term "endophytes" merely refers to the location, that such organisms inhabit. Endophytes include microorganisms such as bacteria, viruses and fungi and these may inhabit different plant tissues internally. Fungal endophytes are very diverse and abundant in all plants examined so far (e.g., Arnold and Lutzoni 2007). One group of endophytes, the epichloid endophytes, lives intercellularly and asymptomatically within aboveground tissues of cool-season grasses in the family Pooideae (Hamilton and Faeth 2005). The epichloid endophytes of genus *Epichloë* (Ascomycota, Clavicipitaceae) and their asexual anamorphs in the genus *Neotyphodium* (Glenn *et al*., 1996) are well known to dramatically alter host plant properties, especially via the production of toxic alkaloids, which in turn, may have consequences for consumer communities (Jani *et al*., 2010).

Epichloë and its anamorph *Neotyphodium* (the epichloid endophytes) exemplify a comprehensive genus of the family Clavicipitaceae (Ascomycota) (Taxonomy comprising of the genus *Epichloë* is complex and is currently in transition) (Oberhofer 2012). The sexual and horizontally transmitted *Epichloë* species form orange-colored external reproductive structures, the stromata, which envelope inflorescences and the upper leaf sheaths of flowering culms and cause choke disease (Leuchtmann 2003). The anamorph of *Epichloë* is classified as *Neotyphodium* (Figure 1) (Glenn et al., 1996). The asexual fungal endophyte *Neotyphodium* systemically infects host grasses, is vertically transmitted by hyphae growing into seeds, and does not cause disease (Jani et al., 2010).

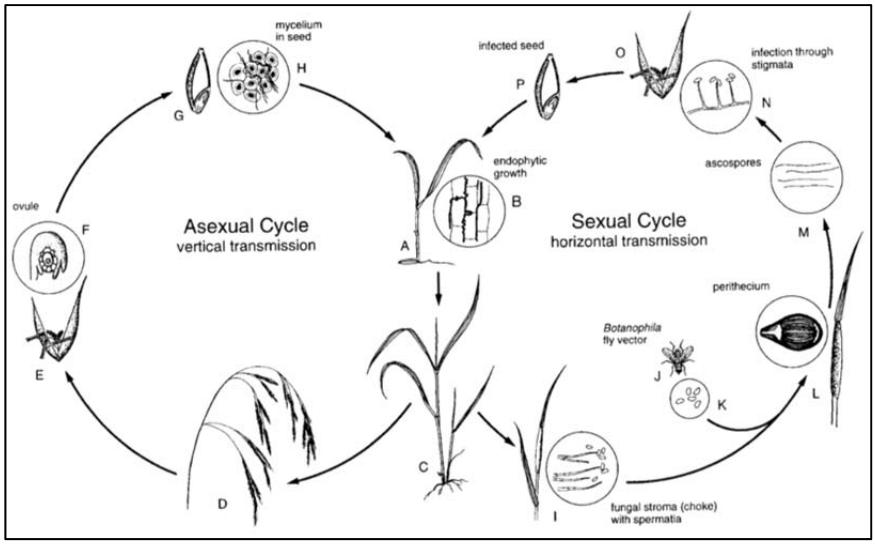

Figure 1. Alternative Life Cycles of an *Epichloë* Species. Displays both the asexual and sexual cycle of the fungus (Leuchtmann and Schardl 1998, Leuchtmann 1993, Clay and Schardl 2002). In epichloid endophytes, the genus *Epichloë* refers to the sexual cycle, while the anamorphic genus *Neotyphodium* refers to the asexual cycle (Oberhofer 2012). Our study focused on the asymptomatic asexual life cycle of *Neotyphodium* sp. During the asexual cycle the endophyte grows in intercellular spaces of above ground tissues of infected grasses during vegetative growth and is

vertically transmitted by entering the ovule of developing inflorescence (Sampson 1933). The sexual cycle begins asymptomatic with an infected grass individual as well. However, when the grass host sets inflorescence, the fungus produces an external structure, the stroma, which replaces plant reproductive tissues. Stromata are heterothallic and carry conidia that serve as spermatia. A fly vector of the genus *Botanophila* sp. fertilizes stromata of opposite mating type by feeding and ovipositing on the stromas surface. Fertilized stromata develop perithecia, in which filamentous ascospores develop. These meiospores are wind-dispersed and infect new hosts by germination on uninfected tissues (Clay and Schardl 2002, Leuchtmann 2003).

Epichloid endophytes are known to have wide-ranging biological effects on the growth and reproduction of their host grasses in cultivated turf and pasture grasses as well as wild grasses (Saikkonen et al., 1998). For example, *Neotyphodium* endophytes infect many cool season pooid grass species and are economically important, particularly because they infect several key agronomic grasses (tall fescue, perennial ryegrass, and annual ryegrass) (Saikkonen et al. 1999, Jani et al. 2010, Bultman et al. 2012). Studies concerning agronomic grasses have led to the theory of defensive mutualism (Clay 1988, Cheplick and Clay 1988). As defensive mutualists, epichloid endophytes may produce alkaloids of four different classes, which are known to act as deterrents specifically against nematodes, insects, mollusks, birds,mammals, mycorrhizal, and pathogenic fungi (Schardl et al., 2004). Because grasses produce few secondary chemicals for defense, endophytes may act as "acquired defenses" (Cheplick and Clay 1988; Clay 1990) via fungal alkaloids that deter or harm herbivores, seed predators and pathogens.

Although increased herbivore resistance is the most renowned effect of endophytes, other reported beneficial effects of epichloid endophytes include drought tolerance, allelopathic effects, increased nutrient uptake, and increased resistant to

oxidative stress, (Kuldau and Bacon 2008, Oberhofer et al., 2013, Cheplick and Faeth 2009). Observed high infection frequencies of *Neotyphodium* sp. in both agronomic and natural grasses are provided as support for the purported mutualism between endophytes and their host grasses (Clay 1998, Faeth 2002).

However, more recent evidence suggests that the interaction between epichloid endophytes is not always mutualistic and depends on endophyte and plant genotypes and on biotic and abiotic factors (e.g., Faeth 2002, Cheplick and Faeth 2009). Variability in interaction outcomes is especially great in wild grasses where plant and endophyte genotypes are diverse and biotic and abiotic factors change spatially and temporally. Studies involving wild grasses are relatively scarce and tend to be limited to short-term investigations (Oberhofer and Leuchtmann 2012). The outcome of endophyte-host grass interactions in these wild grasses varies greatly from mutualism to parasitism. For example, in some cases, endophyte infection may increase herbivore resistance (e.g., Koh and Hik 2007) while in others infection may decrease herbivore resistance (Saikkonen et al., 1999; Tibbets and Faeth 1999; Jani et al., 2010). In Arizona fescue (*Festuca arizonica*), a well-studied wild grass-endophyte system, *Neotyphodium* endophytes may even have negative effects on their host grass, by reducing competitive abilities and decreasing host resistance, at least during certain lifestages of the host (Faeth and Sullivan 2003).

One reason why endophytes may have variable effects on herbivores is that *Neotyphodium* endophytes, especially in wild grasses, harbor genetic variability in alkaloid genes and thus the types of alkaloids produced (Schardl et al. 2012).

Neotyphodium endophytes contain variations in alkaloid genes within and across species. Although asexual, *Neotyphodium* endophytes can acquire rapid infusion of genetic variability by somatically hybridizing with co-occurring epichloid endophytes within the same host grass individual. Recent studies have also shown that hybrid endophytes often harbor more genes for alkaloid synthesis (e.g., Schardl et al. 2012).

A second reason why endophytes may not always interact mutualistically with their hosts, especially in natural populations, is the presence of other trophic levels. In addition to affecting herbivores, alkaloids produced by *Neotyphodium* endophytes may also have negative bottom-up effects on the natural enemies (predators and parasites) of herbivores (Saikkonen et al. 2010, Faeth and Saari 2012). Indeed, just like plant-produced alkaloids, fungal alkaloids may have stronger negative effects on generalist predators than herbivores themselves because, 1) many herbivore species evolve to tolerate or even require plant alkaloids for development, and, 2) some herbivore species may sequester plant allelochemicals for their own defense (e.g., Pasteels 2007, Faeth and Saari 2012).

Thus, supposedly "acquired defenses" (Cheplick and Clay 1988) of grasses via endophytic alkaloids could instead act to decrease resistance, if natural enemies of herbivores are less effective in controlling herbivore populations on infected plants. If so, then endophyte infection has a positive and indirect top-down effect on herbivores through reduced predation. A few studies have shown that endophyte infection and presumably their alkaloids can negatively affect predators (e.g., de Sassi et al. 2006)

and parasites (Omacini et al. 2001, Bultman et al. 1997) and may result in increased herbivore diversity and abundances (Jani et al. 2010).

My study tested the hypothesis that genetic variation of plant endophytic symbionts, including variation caused by hybridization, and consequentially alkaloid levels and types, 1) alters resistance to herbivores and 2) affects preference and performance of natural enemies of the herbivores.

Study Plant Arizona Fescue

Arizona fescue (*Festuca arizonica* Vasey) of the host tribe Poeae is a native North American cool-season, pooid grass species that is commonly infected by epichloid endophytes. Two different endophyte types may infect Arizona fescue: the hybrid endophyte, *Neotyphodium tembladerae*, and the non-hybrid endophyte, *Neotyphodium huerfanum* (Schardl *et al*. 2009). Arizona fescue is native to the southwestern USA and northern Mexico in semi-arid Ponderosa pine-bunch grass communities at elevations between 2,300 m and 3,200 m (Kearney and Peebles 1960; Hamilton and Faeth 2005). Infection rates of this grass species by fungal endophytes can be variable, but are usually high and range from 40-100% of plants in different wild populations (Schulthess and Faeth 1998).

Study Plant Sleepygrass

Achnatherum robustum (Vasey) Barkworth is a native North American cool-season, pooid grass species that is commonly infected by epichloid endophytes.

Achnatherum robustum, commonly known as Sleepygrass, is a perennial bunchgrass native to the southwestern USA and Mexico in semi-arid pine and fir grasslands above 2500 m (Jani *et al*., 2010). Sleepygrass is infected with a hybrid endophyte, *Neotyphodium funkii* of the host tribe Stipeae (Schardl et al. 2009) and another yet unidentified *Neotyphodium* hybrid species (C. Young, personal communication). Infection rates of this grass species by fungal endophytes are variable. Infection frequencies of individual plant tillers of sleepygrass are often high (near 100%), although infection of individuals within a population ranges from 50- 100% (Faeth et al. 2006).

Hybridization of Endophytes

Arizona fescue can be infected by either a hybrid or a non-hybrid strain of *Neotyphodium*, whereas sleepygrass apparently only harbors hybrid endophytes. Although both endophyte types may occur in the same population, they do not co-occur in the same plant. Observations of wild Arizona fescue populations show that generally non-hybrid endophytes are more frequent across populations (Hamilton et al. 2009). This is in contrast to most endophyte-grass systems where hybrid endophytes are more common among grass populations of wild grass species (Oberhofer and Leuchtmann 2012). However, previous studies showed that Arizona fescue hybrid *Neotyphodium* infected grasses (H+) may dominate in resource-poor environments, whereas non-hybrid endophyte-infected (NH+) grasses dominate in environments with more resources (Hamilton et al. 2009, Saari and Faeth 2012).

Hybridization is thought to occur when two different epichloid endophytes infect the same host individual. Fusion of vegetative cells in the hyphae of the two endophytes occurs during the parasexual cycle, resulting in a heterokaryotic state (Figure 1, Schardl and Craven 2003). After fusion, karyogamy then takes place, in which the nuclei fuse and become diploid (n + n = 2n). However, the diploid state is usually unstable and chromosomes are lost at random. Eventually, nuclear genotypes may stabilize at an intermediate (heteroploid) state (Schardl and Craven 2003). Resulting hybrids are asexual (all hybrids are termed *Neotyphodium* species) and thought to be strictly vertically transmitted (the *Epichloë* ancestors may either be transmitted vertically or horizontally). Hybridization is proposed (Schardl and Craven 2003) to enhance fitness of the endophyte in two ways: 1) by masking or reversing the effects of Muller's ratchet (the accumulation of mutations in asexual organisms over time (Selosse and Schardl 2007) or 2) providing genetic variation in alkaloid or other genes to cope with herbivores or other environmental stressors.

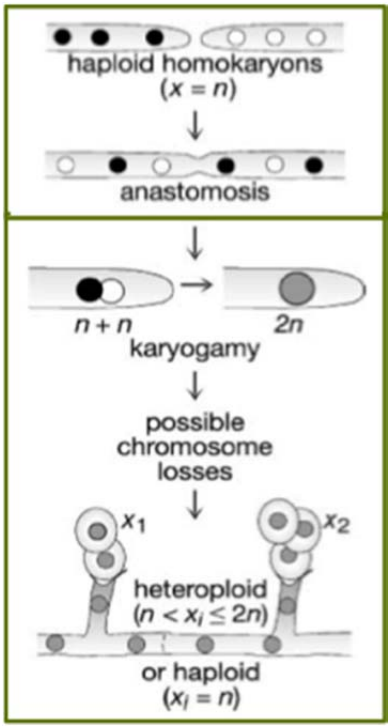

Figure 2. Possible Mechanisms of Vegetative (Somatic, Parasexual) Hybridization in Epichloid Endophytes. Chromosome numbers are indicated as x, with the haploid number = n. In epichloid endophytes hyphae of haploid homokaryons can fuse (anastomosis). If the anastomosing individuals differ genetically, the result is a heterokaryon (hyphal cell containing multiple, genetically different nuclei) or, if mating types differ, a dikaryon (certain fungi having pairs of associated, but unfused haploid nuclei in mycelium prior to their ultimate fusion). Karyogamy (the fusion of pronuclei of two cells), which is a feature of the sexual cycle, may also occur in vegetative heterokaryons; however, the diploid state is usually unstable. Chromosomes are thought to be lost at random. Eventually, nuclear genotypes may stabilize at a haploid or intermediate state (heteroploid), thus, developing what is known as the hybrid form of an endophyte (Schardl and Craven 2003).

Alkaloid Effects on Herbivores

Anti-herbivore properties of *Neotyphodium*-infected host grasses are attributable to the production of diverse biologically active alkaloids by the endophyte (Hamilton and Faeth 2005; Brem and Leuchtmann 2001). Epichloid endophytes can produce many different alkaloids in four classes of alkaloids, each

with varying biological activity against invertebrate and vertebrate herbivores (Leuchtmann *et al*., 2000; Schardl *et al*., 2004; Jani *et al*., 2010). These four classes of alkaloids include: saturated aminopyrrolizidines (lolines), peramine, ergot alkaloids and indolediterpenes (lolitrems) (Bush et al., 1997; Siegel and Bush 1997; Brem and Leuchtmann 2001).

Of those alkaloids, peramine is most commonly found in endophyte-infected host grasses, followed by ergot alkaloids (50%), loline alkaloids (35%), and lolitrems (10%) (Siegel et al.1990). Peramine alkaloids are known to act as a feeding deterrent against insects such as the bird-oat cherry aphid (*Rhopalosiphum padi*) (Siegel et al. 1990), and the Argentine weevil (*Listronotus bonariensis*), but other insects may be insensitive to this alkaloid (Prestidge and Gallagher 1988; Rowan and Latch 1994;Brem and Leuchtmann 2001). Ergot alkaloids and lolitrems are thought to be primarily active against vertebrates. Ergot alkaloids cause a toxicosis syndrome in livestock, while lolitrems are responsible for neurotoxic disorders of mammals (Prestidge 1993). Lolines have a wide range of negative effects on insects, and may also deter small mammal herbivory (Coley et al., 1995; Bush et al., 1997; Brem and Leuchtmann 2001).

Most endophyte studies on mutualism between host grasses and their endophytes have focused on agronomic grasses (Saikkonen et al. 1998). The majority of these studies indicate that endophytes associated with tall fescue (66%) and perennial ryegrass (71%) negatively affect herbivores (Saikkonen 1998). However,

endophyte infection in native grasses have much more variable effects on herbivores, ranging from negative to neutral to positive (e.g., Faeth and Saari 2012). Some studies show that endophytes in native grasses can deter herbivores (Crawford et al. 2010), while others such as Faeth (2009) and Jani et al. (2010) found higher abundances of invertebrate herbivores on endophyte-infected Arizona fescue and Sleepygrass. This variation in endophyte effects on herbivores may depend on plant and endophyte genotypic variation and environmental factors, as well as the herbivore species used in assays (Cheplick and Faeth 2009).

Endophytic Alkaloids Also Affect Herbivore Enemies

Alkaloids produced by endophytes are well known to negatively affect some herbivores. However, it is yet not well understood, if alkaloids can also cascade up the food web and affect the third trophic level, predators and parasites of herbivores (Faeth and Bultman 2001, Bultman et al. 2012, Faeth and Saari 2012, Saari et al. 2013). Recent studies show that alkaloids can be transferred from plant to aphid herbivore to insect predator (Fuchs et al. 2013). Thus, one explanation for the variable results of endophyte-herbivore interactions in native grasses is that alkaloids may have a negative effect on natural enemies of herbivores (Faeth and Saari 2012). If so, then the defensive mutualism may not hold true if the negative effects on natural enemies outweighs those on the herbivores.

Previous studies show that alkaloids could be sequestered by herbivores to deter their natural enemies and thus resulting in increase herbivore abundance (de

Sassi et al., 2006, Faeth and Saari 2012; Jani et al., 2010, Fuchs et al. 2013) and counteracting the notion that endophytes act as defensive mutualists. Alternatively, endophyte infections may inhibit herbivores from inducing defenses against their predators, rendering them more susceptible to predation (Zust et al. 2009). Therefore, cascading alkaloids to higher trophic levels could result in either reduced or increased herbivore loads, and consequently either enhance or diminish the efficacy of endophytic alkaloids as acquired defenses of the host grass (Figure 3).

In addition, specialist herbivores may be less affected by endophytic alkaloids because they have evolved to detoxify plant and endophytic allelochemicals (Faeth and Saari 2012), and even use them as their defense against their natural enemies. This mechanism could hold for specialist insect herbivores that co-evolved with the grass and might be able to detoxify the alkaloids, or even require plant allelochemicals for oviposition and survival, and use them as defense against natural enemies (Faeth and Saari 2012).

Hypotheses

1) Endophytic alkaloids affect aphid herbivore abundances on two species of native grasses. Endophyte effects on aphids depend on the genotype of the endophyte and the types of alkaloids that are produced.
2) Endophyte infection and associated alkaloids affect not only aphid performance but also choice when selecting plants for feeding.

3) Endophytic alkaloids cascade through the food web, affecting the third trophic level, predators of aphid herbivores by altering development time and feeding preferences. Endophyte effects on predators depend on the genotype of the endophyte and the types of alkaloids that are produced.

I tested these hypotheses using Arizona fescue (*Festuca arizonica*) and sleepygrass (*Achnatherum robustum*) as the host plants, the bird cherry-oat aphid (*Rhopalosiphum padi*) as the herbivore and the ladybird beetle (*Coleomegilla maculata*) as the natural enemy in experiments and observations.

Predictions

1) Bird oat cherry aphids reared on infected Arizona fescue should have lower abundance and reproduction and thus lower population sizes than aphids reared on uninfected Arizona fescue. Aphids reared on Arizona fescue infected by hybrid endophytes should have lower population sizes than aphids reared on Arizona fescue infected by non-hybrid endophytes. Likewise, aphids reared on sleepygrass infected by endophytes that produce ergot alkaloids should have lower population sizes than those reared on sleepygrass without ergot alkaloids.
2) Aphids should prefer to feed on Arizona fescue in the following order: uninfected (E-) > non-hybrid infected (NH+) > hybrid infected (H+) plants.

For sleepygrass, aphids should prefer plants without ergot alkaloids relative to infected plants with ergot alkaloids.

3) If endophytic alkaloids cascade upward to higher trophic levels, then ladybird beetles feeding on aphids from hybrid infected plants should have slower development times than those feeding on aphids reared on non-hybrid infected and uninfected Arizona fescue. Lady bird beetles should prefer to feed on aphids reared on Arizona fescue in the following order: uninfected (E-) > non-hybrid infected (NH+) > hybrid infected (H+) plants.

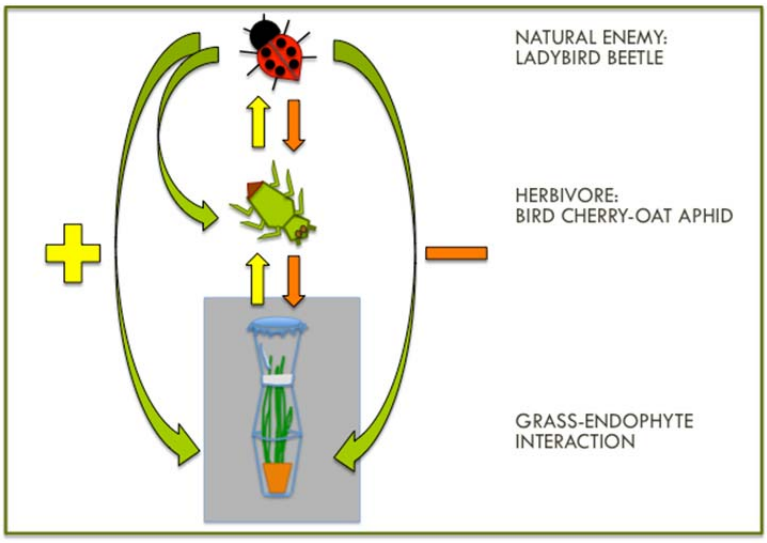

Figure 3. Multitrophic Interactions. The presence of alkaloids can be either beneficial to the plant, negatively affect the plant, or remain neutral. In the negative feedback cycle the presence of alkaloids by endophyte infection cascades up the food chain whereby the aphids sequester the alkaloids for their own defense. In this scenario, number of the aphid predators, the ladybird beetle, is negatively affected by alkaloid presence thus lowering ladybird numbers and negatively affecting the plant. In the positive feedback cycle, the aphid is negatively affected by alkaloid presence, but the ladybird beetle can sequester the alkaloids for their defense, thus, lowering aphid numbers and benefiting the host plant.

CHAPTER II

MATERIALS AND METHODS

Alkaloid Analyses

Endophyte infection in Arizona fescue by hybrid and non-hybrid endophytes is known to produce peramine as the primary alkaloid type (Faeth et al. 2002, Schardl et al. 2011). However, recent evidence indicates that both hybrid and non-hybrid *Neotyphodium* endophytes in Arizona fescue may also harbor genes for indolediterpene alkaloids; although it is yet unclear if actual indoledipterpenes are produced (Saari et al. 2013). Peramine concentrations of a random sample of five plants from all infection categories (E-, NH+, H+, NH-, H-, see below) (N = 25) were analyzed by J. Strickland, University of Kentucky, USDA-ARS, using the method described in Faeth et al (2002) with concentrations ranging from 3 – 12 ppm. Endophytes of sleepygrass individuals are known to produce at least ergot alkaloids (Lysergic acid amide (LAA) and ergonovine) (Faeth et al 2006). Ergot alkaloid concentrations were based on assays of a random sample of 56 plants and were analyzed by A. Jarmusch and N. Cech, University of North Carolina at Greensboro, Chemistry & Biochemistry Department, using an HPLC-HESI-MS method (unpublished data). Ergot alkaloid levels ranged from 26.74 to 252.14 ppm.

Effect of Endophyte Infection in Sleepygrass on Bird Cherry-Oat Aphid Populations

To test the effects of endophyte-infected sleepygrass on numbers of the bird cherry-oat aphid, aphids were reared on two ergot alkaloid positive (populations 4 and 20) and nine ergot alkaloid negative sleepygrass plants (populations 1, 2, 3, 5, 6, 7, 8, 9, T, and W). I used two different strains of bird oat cherry aphids that were thought to vary in sensitivity to ergot alkaloids (ergonovine and lysergic acid amide). One strain (NY) originated from the state of New York and was known to be tolerant to high ergot alkaloid levels within grasses (pers. comm., Megan Rua and Marty Dekkers, UNC-Chapel Hill). The other population (NC) originated from the greenhouse of the University of North Carolina Greensboro Biology Department. This population was presumably sensitive to endophytic ergot alkaloids based upon observations of greenhouse populations.

The experiment began in October 2011 and ended in December 2011. The randomized block design of the experiment consisted of two plant types. Ergot-positive plants had high ergot alkaloid levels ranging from 26.74 to 252.14 ppm. Ergot-free plants did not contain ergot alkaloids (other alkaloids such as peramines or indoleterpenes were not tested). Ergot alkaloid sensitive (NC) and tolerant (NY) aphid strains were reared on one alkaloid and one alkaloid-free plant, respectively replicated 14 times (14 blocks), for a total of 56 plants (2 aphids types*2 plant types*14 blocks = 56).

The experiment was started with three aphids that were initially transferred onto each plant and were then enclosed in a well-ventilated clear container (Figure 4).

Numbers of aphids were recorded every three days. To maintain a measureable population on all plant individuals, three additional aphids were added to each plant every three days. Sleepygrass plants were given 1.23 ml of fertilizer (ratio of 20:20:20 for nitrogen, phosphate, and potassium) per 1.66 liters of water once a week and watered with 50 ml tap water three times a week. Plants were kept in a growth chamber with a day/night cycle of 8/16 h and a constant temperature of 22 ° C.

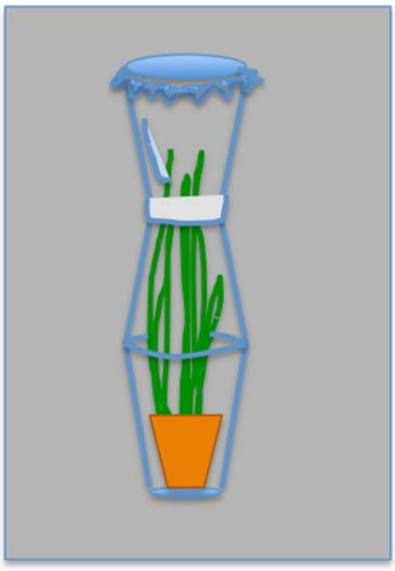

Figure 4. Design of Aphid Feeding Experiment Enclosure. Depending on the experiment, this enclosure design was used for either Arizona fescue or Sleepygrass, and always contained a single plant. Aphids were reared on potted grass and enclosed in a clear container. The container consisted of three clear cups (Solo Cup Company) held together by Parafilm. The top of the container was closed using thin sheer light-blue fabric and secured with rubber bands.

Effects of Hybrid, Non-Hybrid and Uninfected Arizona Fescue on Bird Cherry-Oat Aphid Abundance

We tested the effects of hybrid and non-hybrid endophytes in Arizona fescue on the abundance of NC and NY populations of bird cherry-oat aphid. Hybrid endophytes may confer greater resistance against herbivores than non-hybrid endophytes because of increased number of alkaloid genes, resulting in more diverse alkaloids (e.g., Schardl and Craven 2003). Five infection categories of plants were used to examine this hypothesis: non-hybrid infected (NH+), hybrid infected (H+), and naturally endophyte-free (E-) plants based on their original infection status in natural populations. Further, we used plants of two additional types, from which endophytes had been experimentally removed: originally hybrid endophyte hosting plants (H-) and non-hybrid endophyte hosting plants (NH-). Details of experimental endophyte removal can be found in Saari et al. (2013). Seeds from each of the five infection categories were germinated, planted, and grown in the laboratory. Seeds from five different plant maternal genotypes within each infection category were used to randomize plant genotype effects.

Two aphid strains and five plant infection categories were examined in a factorial design and arranged in twenty blocks as replicates (randomized block design, 2 aphid strains *5 infection types *20 blocks = 200). The experiment was carried out for one month in April 2012. Initially, three aphids were placed onto each plant. Numbers of aphids were counted on each plant every three days. After each counting event, three additional aphids were placed on each plant to maintain viable

populations on all plants (some plants had no aphids after the original placement of three aphids). Position of plants were randomized and maintained in a growth chamber with growth conditions as described above. At the end of the experiment, the total number of aphids per plant was recorded.

Bird Cherry-Oat Aphid Feeding Preference

Aphid feeding preferences on the five infection categories ((non-hybrid infected (NH+), hybrid infected (H+), naturally endophyte-free (E-), experimentally removed hybrid (H-) and non-hybrid endophyte hosting plants (NH-)) were examined by eight pairwise choice tests as illustrated in Fig. 5 (9 blocks x 8 infection category comparisons = 72 petri dishes). For each pairwise comparison, one tiller from each corresponding infection category was placed symmetrically at equal distances from the edge of the petri dish (Figure 6). Petri dishes were kept at room temperature in the laboratory and placed on the laboratory bench. Aphids used in this experiment had been reared on oats as a neutral diet that contains no known alkaloids. One aphid was selected randomly and placed centrally into a petri dish and allowed to roam freely and choose between the two experimental tillers of different infection categories (Figure 6). The choice of each aphid was recorded after 1 and 5 hours. If aphids did not choose between either of the two tillers, the preference was recorded as 0.

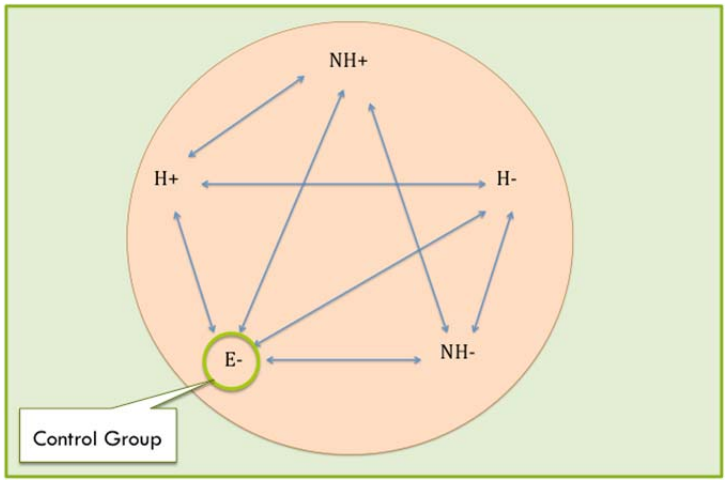

Figure 5. Design of the Dual Aphid Feeding Choice Preferences. Arrows indicate the dual choice options presented to the aphids covering each dual combination of infection categories with E- serving as control.

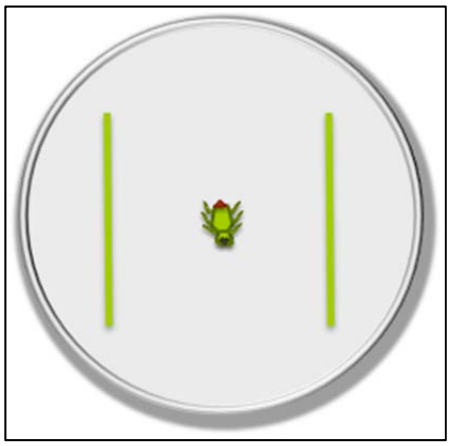

Figure 6. Experimental Setup of a Single Aphid Feeding Choice Trial. Inside the petri dishes, one tiller from a pair of infection categories was placed symmetrically at equal distances from the edge of the petri dish. The pairwise combinations are as shown in Figure 5.

Effects of Infection Status and Type on the Development of Ladybird Beetles

I tested the effect of endophyte infection categories in Arizona fescue on a predator of the bird cherry-oat aphid, the ladybird beetle, *Coleomegilla maculata*. The experimental design consisted of one aphid strain (NC), five infection categories (NH+, H+, E-, NH- and H-) and was replicated in seven blocks (1 ladybird *5 infection categories *7 blocks = 35). Eggs of female ladybird beetles were obtained from the laboratories of Dr. Lundgren, USDA/ARS North Central Agricultural research laboratory, Brookings, SD, USA and raised until adulthood in the lab. Ladybird eggs were transferred onto a petri dish and remained in the growth chamber with a day/night cycle of 8/16 h and a constant temperature of 22 ° C, until they hatched.

Once hatched, ladybird larvae were initially weighed. One larva was then placed on each Arizona fescue plant. At least 50 aphids were placed into each well-ventilated clear container to guarantee *ad libitum* prey availability for the larvae (Figure 4). Individual ladybird larvae were allowed to feed on the aphids. Each larva was weighed every three days and development time was monitored through adult stage. Adult ladybird beetles were then kept in the growth chamber with the same conditions as described above.

All adult beetles that survived were later used in the second generation experiment (see below) and were fed Lundgren's Super C MAC Diet of artificial food: (five parts Bee-Pro pollen substitute (Mann Lake Ltd, MN, USA), three parts tropical fish flakes (Tetra Color TM tropical fish flakes, Tetra Holding Inc.,

Blacksburg, VA, USA), three parts cichlid pellets (Omega One cichlid pellets, Omega Sea, Ltd, Sitka, AK, USA), three parts sun-dried *Gammarus pulex* ((L.) (Amphipoda: Gammaridae) (Tetra Baby Shrimp, Tetra Holding Inc.)), and two parts *Ephestia kuehniella* eggs (Beneficial Insectary, Redding, CA, USA). All components (aside from the eggs) were pulverized using a mortar and pestle prior to mixing the diet. Beetles received tap water by a saturated square piece of sponge 5 cm^2 and a drop of honey was placed on the petri dish lid. The diet was refreshed every 2-3 days and petri dishes were cleaned as needed.

Ladybird Beetles Feeding Preference Experiment

For this experiment, Arizona fescue plants that contained bird cherry-oat aphids from the ladybird beetle development experiment (first generation) were re-used. The design was 8 pairwise comparisons of the five different infection categories (NH+ vs. NH-, NH+ vs. E-, NH- vs. E-, H+ vs. H-, NH+ vs. H+, NH- vs. H-, E- vs. H+, and E- vs. H- (same categories were not compared) (Figure 5). To focus on hybrid vs. endophyte comparisons of similar types, infection categories NH- vs. H+ and NH+ vs. H- were not compared. Each pairwise comparison was replicated twenty times (8 infection category comparisons *20 replicates= 160 plant comparisons). Two Arizona fescue plants of different infection category that were similar in height and number of tillers were used in each trial.

The experiment was conducted as a double blind design. Plant pairs were chosen at random without the experimenter's knowledge of infection category. Plants

were placed in a box to decrease the possibility of outside environmental influences on ladybird beetle preference 4 cm apart of each other. For each replicate, a ladybird beetle was placed in between the two plants in a small clear petri dish. Each of the 20 replicates was performed with a naïve individual ladybird beetle originating from the same population. All ladybird beetles that were used in the experiment were originally fed Lundgren's Super C MAC diet (see above)

Ladybird beetle choice was determined when the beetle made contact with the tillers of a particular plant. The time from opening of the lid of the dish to when the ladybird beetle chose a plant was recorded.

Effects of Grass Infection Status and Type on the Development of Second-Generation Ladybird Beetles

I monitored the development of second-generation ladybird beetles originating from first generation adult ladybird beetles used in the choice test that were mated with first generation ladybird beetles fed with Lundgren's Super C MAC diet. I used one adult beetle of random gender from the choice test per infection category and paired them with one adult beetle that was not part of the choice experiment of the opposite gender and had seven replicates in each group (1 mating pair *5 infection categories *7 replicates = 35). To identify gender, all of the first generation ladybird beetles were temporarily anesthetized using carbon dioxide. All beetles that were fed a diet of bird cherry-oat aphids were marked with a blue dot of paint on the elytra and were contained individually in petri dishes. The ladybird beetles that were not used

during the choice experiment and fed Lundgren's Super C MAC diets were separated based on gender in clean, clear and ventilated containers, but no individuals were marked. All beetles were maintained in the growth chamber as described above in the methods of the ladybird beetle choice experiment.

Ladybird beetles that had been in the choice experiment were then mated with the ladybird beetles originally fed a diet of Lundgren's Super C MAC diet by matching opposite sexes. Each choice experiment ladybird beetle was mated sequentially from the date that they had originally hatched. Any ladybird beetle that did not mate was given a new partner (from the corresponding diet) the next day. Eggs were counted and transferred onto new petri dishes. Hatching larvae were maintained like the adult beetles in the growth chamber (see methods above). Larvae originating from each mating event were counted. Development of the ladybird beetles from egg to adulthood was monitored. I recorded the number of days of development, total number of adults, proportion of males to females, and average weight of each second generation ladybird beetle based on infection category of the grass that was the nutritional source of the aphids, which one of the parental beetles had consumed.

CHAPTER III

DATA ANALYSIS

Effect of Endophyte Infection in Sleepygrass on Bird Cherry-Oat Aphid Populations

I used a univariate generalized linear model (GLM) in SPSS 20.0.0 (IBM SPSS, Chicago, USA) to analyze the effect of alkaloid status (A+, A-) on the total number of two populations of wingless aphids (NY, NC) that survived 8 weeks (10/28/2013 – 12/20/2013) until the end of the experiment. The dependent variable was the total number of wingless aphids. The experiment was conducted as a random block design and the statistical model included alkaloid status as a fixed effect. All tests were based on a confidence interval of 95%.

Effects of Hybrid, Non-Hybrid and Uninfected Arizona Fescue on Bird Cherry-Oat Aphid Abundance

Pairwise comparisons of the total number of aphids were performed with a univariate analysis using a general linear model with infection status and aphid population as fixed factors. Data testing the difference in aphid abundance between NY and NC aphid populations were analyzed using a binary logistic regression, in which aphid population, block, and infection status were included as covariates (SPSS 20.0.0 (IBM SPSS, Chicago, USA)).

Bird Cherry-Oat Aphid Feeding Preference

A discrete choice conditional logit model (Train, 2009) was used with SAS Statistics 2012 (SAS, Cary, NC USA) to assess aphid preferences choosing between two tillers with different endophyte categories.

Effects of Infection Status and Type on the Development of Ladybird Beetles

Ladybird development and weight were analyzed with a univariate analysis using a general linear model in SPSS 20.0.0 (IBM SPSS, Chicago, USA). Ladybird beetle development was dependent on the plant's infection category, which was treated as a fixed factor and block as a random factor. All tests were based on a confidence interval of 95%.

Ladybird Beetles Feeding Preference Experiment

A discrete choice conditional logit model (Train, 2009) was used with SAS Statistics 2012 (SAS, Cary, NC USA) to assess ladybird preferences for choosing between two plants with different endophyte categories. In addition to feeding preference in reference to endophyte infection categories, the number of aphids, number of tillers, plant height, and time were also investigated (Richter 2013).

Effects of Grass Infection Status and Type on the Development of Second-Generation Ladybird Beetles

Ladybird adult survival, development and weight were analyzed with SPSS 20.0.0 (IBM SPSS, Chicago, USA) using a univariate general linear model. Infection category was treated as a fixed factor and block as a random factor. All tests were based on a confidence interval of 95%.

CHAPTER IV

RESULTS

Effect of Endophyte Infection in Sleepygrass on Bird Cherry-Oat Aphid Populations

The presence of ergot alkaloids in endophyte-infected sleepygrass plants reduced aphid population size ($F(2, 53) = 6.34$, $p = .003$, Table 1). Collectively (NC and NY strains combined), the mean number of aphids on sleepygrass without ergot alkaloids was higher (mean = 74.32, SE = 15.42) than the mean number of aphids on sleepygrass with ergot alkaloids present (mean = 11.96, SE = 8.24) (Table 2). For the GLM analysis, the strength of the relationship between ergot alkaloid presence and the number of aphids was large with ergot presence accounting for 19% of the variance of the dependent variable (Table 1). However, there were no differences ($p = 0.699$) between the number of aphids for NY and NC populations, regardless of ergot alkaloid presence (Table 1).

Table 1. GLM Analysis on the Effects of Ergot Alkaloid Presence on the Number of Aphids. The factor "Aphids" represents effects of the two strains of NY and NC aphids. Only data for the month November were analyzed.

Tests of Between-Subjects Effects

Dependent Variable: Aphids

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	55096.071[a]	2	27548.036	6.338	.003
Intercept	104233.143	1	104233.143	23.981	.000
Alkaloids	54437.786	1	54437.786	12.525	.001
Aphids	658.286	1	658.286	.151	.699
Error	230360.786	53	4346.430		
Total	389690.000	56			
Corrected Total	285456.857	55			

a. R Squared = .193 (Adjusted R Squared = .163)

Table 2. Descriptive Statistics for the Number of NY and NC Aphids (Collectively) Based on Ergot Alkaloid Presence.

Descriptives

Aphids		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
						Lower Bound	Upper Bound			
Alkaloids Not Present		28	74.3214	81.58434	15.41799	42.6863	105.9565	.00	313.00	
Alkaloids Present		28	11.9643	43.59195	8.23810	-4.9389	28.8675	.00	209.00	
Total		56	43.1429	72.04252	9.62709	23.8497	62.4360	.00	313.00	
Model	Fixed Effects			65.40742	8.74043	25.6193	60.6664			
	Random Effects				31.17857	-353.0185	439.3042			1791.41624

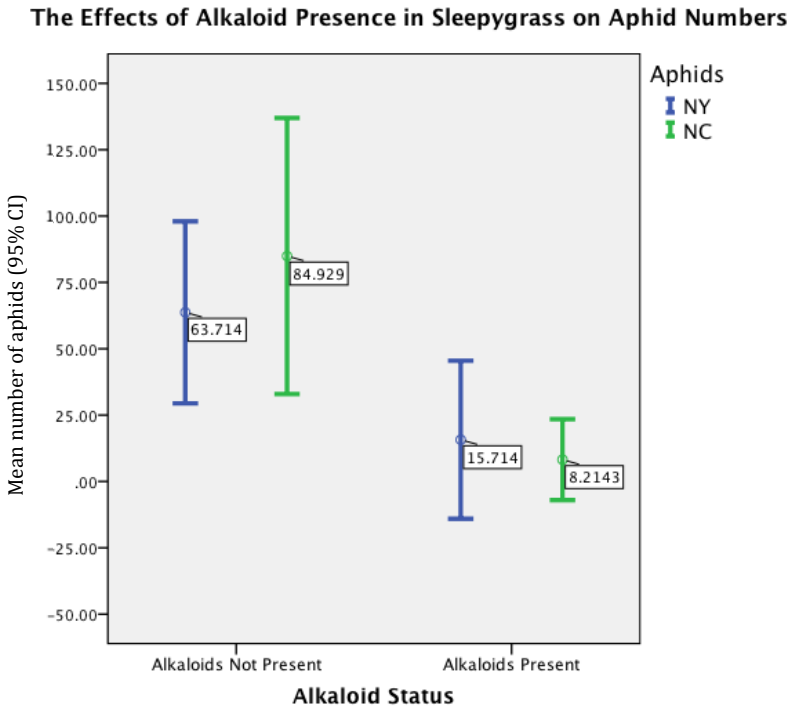

Figure 7. Effect of Alkaloid Presence in Sleepygrass on Bird Cherry-Oat Aphid Numbers. Mean number (with 95% confidence interval) of bird cherry-oat aphid on sleepygrass with and without ergot alkaloids.

Although the presence of ergot alkaloids did not significantly affect the number of aphids, there was a steeper decline in NC aphids feeding on ergot positive (A+) sleepygrass plants than NY aphids (Figure 7). Using a pairwise comparison (LSD post-hoc test), NC aphid numbers separately showed a significant decline on A+ relative to A- plants (p = .001, mean difference = 62.4, 95% CI = 27.016 – 97.698) (Table 3). Furthermore, a GLM analysis revealed a lower number of NC aphids (mean = 8.21, SE = 7.62) on ergot positive plants than on ergot alkaloid free plants (mean = 84.93, SE = 26.00), F (1, 26) = 8.02, p = .009 (Table 4 and 5). The effect of ergot alkaloid presence on the number of NC aphids, as assessed by η^2 was

strong, with ergot alkaloid presence accounting for 24% of the variance of the dependent variable.

Table 3. Pairwise Comparisons (LSD Post-Hoc Test) of the Effect of the Presence of Ergot Alkaloid on the Number of NC Aphids.

Pairwise Comparisons

Dependent Variable: Aphids

(I) Alkaloids	(J) Alkaloids	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Alkaloids Not Present	Alkaloids Present	62.357*	17.620	.001	27.016	97.698
Alkaloids Present	Alkaloids Not Present	-62.357*	17.620	.001	-97.698	-27.016

Based on estimated marginal means

*. The mean difference is significant at the

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 4. The Effect of Alkaloid Presence (GLM Analysis) on the Number of NC Aphids on Sleepygrass.

Tests of Between-Subjects Effects

Dependent Variable: November_Survival_For_NC_Aphids

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	41195.571[a]	1	41195.571	8.015	.009
Intercept	60729.143	1	60729.143	11.816	.002
Alkaloids	41195.571	1	41195.571	8.015	.009
Error	133633.286	26	5139.742		
Total	235558.000	28			
Corrected Total	174828.857	27			

a. R Squared = .236 (Adjusted R Squared = .206)

Table 5. Descriptive Statistics for the Number of NC Aphids Based on Ergot Alkaloid Presence.

Descriptives

November_Survival_For_NC_Aphids

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
						Lower Bound	Upper Bound			
Alkaloids Absent		14	84.9286	97.29611	26.00348	28.7515	141.1057	.00	313.00	
Alkaloids Present		14	8.2143	28.51229	7.62023	-8.2482	24.6768	.00	107.00	
Total		28	46.5714	80.46827	15.20707	15.3691	77.7738	.00	313.00	
Model	Fixed Effects			71.69199	13.54851	18.7221	74.4208			
	Random Effects				38.35714	-440.8023	533.9451			2575.41641

Effects of Hybrid, Non-Hybrid and Uninfected Arizona Fescue on Bird Cherry-Oat Aphid Abundance

A binary logistic regression was performed to test differences in aphid presence between NY and NC aphid populations. There were no differences in aphid presence between the NC and NC aphid populations (p =.163) (Table 6). A pairwise comparison (LSD post-hoc test) was performed to evaluate the relationship between aphid numbers and infection status. Total aphid numbers (NY and NC combined) differed by infection categories, with a higher number of aphids on NH+ (p =.046), NH- (p =.003), and H- (p =.000) plants compared to H+ plants (Table 7). Generally, NY and NC aphid populations were lower on H+ plants than any other plant infection category (Figure 8). No other pairwise comparisons were significant.

When NC and NY aphids were considered separately, NY aphid population sizes were lower on H+ Arizona fescue plants compared to NH+ plants. However, both NY and NC aphids on H+ plants were lower than NY and NC aphids on NH- plants. Generally, NY and NC aphid populations were lower on H+ plants than any other plant category (Figure 8).

Table 6. Binary Logistic Regression to Test the Difference of Aphid Presence Between NY and NC Aphid Populations Feeding on Arizona Fescue.

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1[a]	Aphid	.437	.313	1.942	1	.163	1.548
	Block	.027	.027	1.021	1	.312	1.028
	Category	.206	.112	3.414	1	.065	1.229
	Constant	-1.979	.522	14.390	1	.000	.138

a. Variable(s) entered on step 1: Aphid, Block, Category

Table 7. LSD Post-Hoc Test of Infection Categories on Aphid Population Sizes.

Pairwise Comparisons

Dependent Variable: Individuals

(I) Category	(I)	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
E-	NH+	-.275	1.131	.808	-2.496	1.946
	NH-	-.775	1.066	.468	-2.869	1.319
	H+	1.325	1.032	.200	-.703	3.353
	H-	-1.000	1.012	.323	-2.987	.987
NH+	E-	.275	1.131	.808	-1.946	2.496
	NH-	-.500	.843	.553	-2.156	1.156
	H+	1.600*	.800	.046	.029	3.171
	H-	-.725	.773	.348	-2.242	.792
NH-	E-	.775	1.066	.468	-1.319	2.869
	NH+	.500	.843	.553	-1.156	2.156
	H+	2.100*	.705	.003	.715	3.485
	H-	-.225	.674	.739	-1.549	1.099
H+	E-	-1.325	1.032	.200	-3.353	.703
	NH+	-1.600*	.800	.046	-3.171	-.029
	NH-	-2.100*	.705	.003	-3.485	-.715
	H-	-2.325*	.619	.000	-3.542	-1.108
H-	E-	1.000	1.012	.323	-.987	2.987
	NH+	.725	.773	.348	-.792	2.242
	NH-	.225	.674	.739	-1.099	1.549
	H+	2.325*	.619	.000	1.108	3.542

Based on estimated marginal means

*. The mean difference is significant at the

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

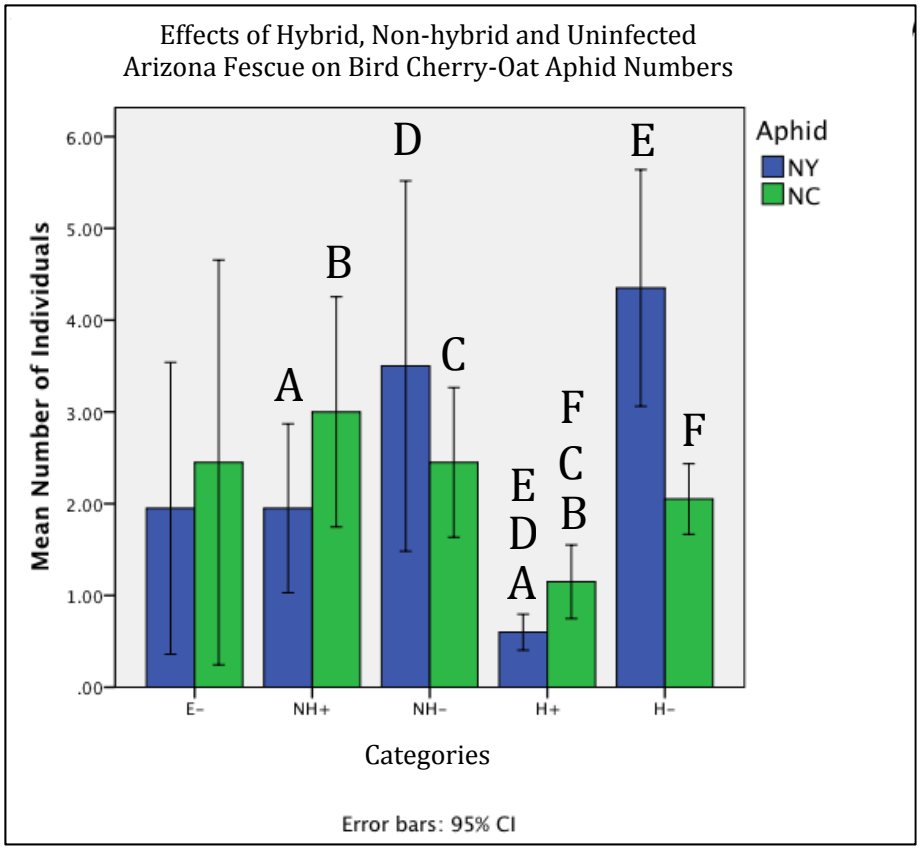

Figure 8. Effects of Hybrid, Non-Hybrid and Uninfected Arizona Fescue on Bird Cherry-Oat Aphid Numbers. Bars (± SD) represent mean aphid numbers of both aphid populations (NY = New York aphid population, NC = North Carolina aphid population). Significant differences between groups are noted with an alphabetic letter: (A, D, E: NY aphid numbers are lower on H+ plants than on NH+, NH-, and H- plants; B, C, F: NC aphid numbers are lower on H+ plants than on NH+, NH-, and H- plants (refer also to Table 7).

Bird Cherry-Oat Aphid Feeding Preference

The type of endophyte affected host plant preference of aphids (Table 8). Generally, aphids avoided H+ plants relative to other plant types. The odds ratio of aphids choosing NH+ plants was more than two times higher than aphids choosing H+ plants (p = .06, Table 8). Similarly, the odds ratio of aphids choosing E- plants was more than three times higher than aphids choosing H+ plants (p = .09, Table 8).

No other pairwise comparisons were statistically significant. It should be noted that odds reported here represent the overall odds of choosing a particular infection category across all alternatives with which it was paired, and not the specific odds of choosing a particular category within a pair (Saari et al. 2013).

Table 8. Odds Ratios Comparing the Odds of Aphid Feeding Preference. Comparing endophyte category 1, over all pairs in which category 1 appeared, to the odds of choosing category 2, over all pairs in which category 2 appeared.

Category 1	Category 2	Odds Ratio (Category 1 / Category 2)	P-value
NH+	H+	3.66	0.059
E-	H+	3.03	0.086
H-	H+	1.84	0.392
NH+	H-	1.99	0.401
E-	H-	1.65	0.500
NH-	H-	1.46	0.628
NH+	NH-	1.37	0.682
NH+	E-	1.21	0.783
E-	NH-	1.13	0.877

Effects of Infection Status and Type on the Development of Ladybird Beetles

Ladybird beetle weight was not affected overall by feeding on aphids from plants in different infection categories, (between-subject effects) F (4, 30) = 1.21, p = 0.326, except for the comparison between E- vs. H, p = .076, in which there was a trend in higher weight for ladybird beetles on E- plants than on H+ plants (Table 10,

Figure 9). Likewise, infection category overall did not affect ladybird beetle development (between-subject effects F (4, 30) = 1.55, p = 0.212 (Table 11). Although not significant, mean development time of ladybirds feeding on aphids from H+ was longer than when feeding on aphids from all other plant categories (Figure 10). For pairwise comparisons, ladybirds feeding on aphids from E- plants developed slower than ladybirds on H- plants (p = .03,Table 12, Figure 10).

Table 9. GLM Analysis of Arizona Fescue Infection Category on Ladybird Beetle Weight.

Tests of Between-Subjects Effects

Dependent Variable: Weight

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.003[a]	4	.001	1.213	.326
Intercept	.008	1	.008	14.424	.001
Infection Category	.003	4	.001	1.213	.326
Error	.017	30	.001		
Total	.028	35			
Corrected Total	.020	34			

a. R Squared = .139 (Adjusted R Squared = .024)

Table 10. LSD Post-Hoc Test of Infection Categories on Ladybird Beetle Weight.

Pairwise Comparisons

Dependent Variable: Weight

(I) Category	(I) Category	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
E-	NH+	-.001	.013	.932	-.027	.025
	NH-	-.003	.013	.800	-.029	.023
	H+	-.023	.013	.076	-.049	.003
	H-	-.001	.013	.929	-.027	.025
NH+	E-	.001	.013	.932	-.025	.027
	NH-	-.002	.013	.867	-.028	.024
	H+	-.022	.013	.090	-.048	.004
	H-	.000	.013	.997	-.026	.026
NH-	E-	.003	.013	.800	-.023	.029
	NH+	.002	.013	.867	-.024	.028
	H+	-.020	.013	.124	-.046	.006
	H-	.002	.013	.870	-.024	.028
H+	E-	.023	.013	.076	-.003	.049
	NH+	.022	.013	.090	-.004	.048
	NH-	.020	.013	.124	-.006	.046
	H-	.022	.013	.091	-.004	.048
H-	E-	.001	.013	.929	-.025	.027
	NH+	4.286E-005	.013	.997	-.026	.026
	NH-	-.002	.013	.870	-.028	.024
	H+	-.022	.013	.091	-.048	.004

Based on estimated marginal means

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 11. GLM Analysis of Arizona Fescue Infection Categories on Ladybird Beetle Development.

Tests of Between-Subjects Effects

Dependent Variable: Development

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	139.143[a]	4	34.786	1.553	.212
Intercept	16502.857	1	16502.857	736.735	.000
Infection Category	139.143	4	34.786	1.553	.212
Error	672.000	30	22.400		
Total	17314.000	35			
Corrected Total	811.143	34			

a. R Squared = .172 (Adjusted R Squared = .061)

Table 12. LSD Post-Hoc Test on Effects of Infection Categories in Arizona Fescue on Ladybird Beetle Development.

Pairwise Comparisons

Dependent Variable: Development

(I) Category	(I) Category	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
E-	NH+	-4.571	2.530	.081	-9.738	.595
	NH-	-2.429	2.530	.345	-7.595	2.738
	H+	-2.857	2.530	.268	-8.024	2.309
	H-	-5.857*	2.530	.028	-11.024	-.691
NH+	E-	4.571	2.530	.081	-.595	9.738
	NH-	2.143	2.530	.404	-3.024	7.309
	H+	1.714	2.530	.503	-3.452	6.881
	H-	-1.286	2.530	.615	-6.452	3.881
NH-	E-	2.429	2.530	.345	-2.738	7.595
	NH+	-2.143	2.530	.404	-7.309	3.024
	H+	-.429	2.530	.867	-5.595	4.738
	H-	-3.429	2.530	.185	-8.595	1.738
H+	E-	2.857	2.530	.268	-2.309	8.024
	NH+	-1.714	2.530	.503	-6.881	3.452
	NH-	.429	2.530	.867	-4.738	5.595
	H-	-3.000	2.530	.245	-8.167	2.167
H-	E-	5.857*	2.530	.028	.691	11.024
	NH+	1.286	2.530	.615	-3.881	6.452
	NH-	3.429	2.530	.185	-1.738	8.595
	H+	3.000	2.530	.245	-2.167	8.167

Based on estimated marginal means

*. The mean difference is significant at the

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

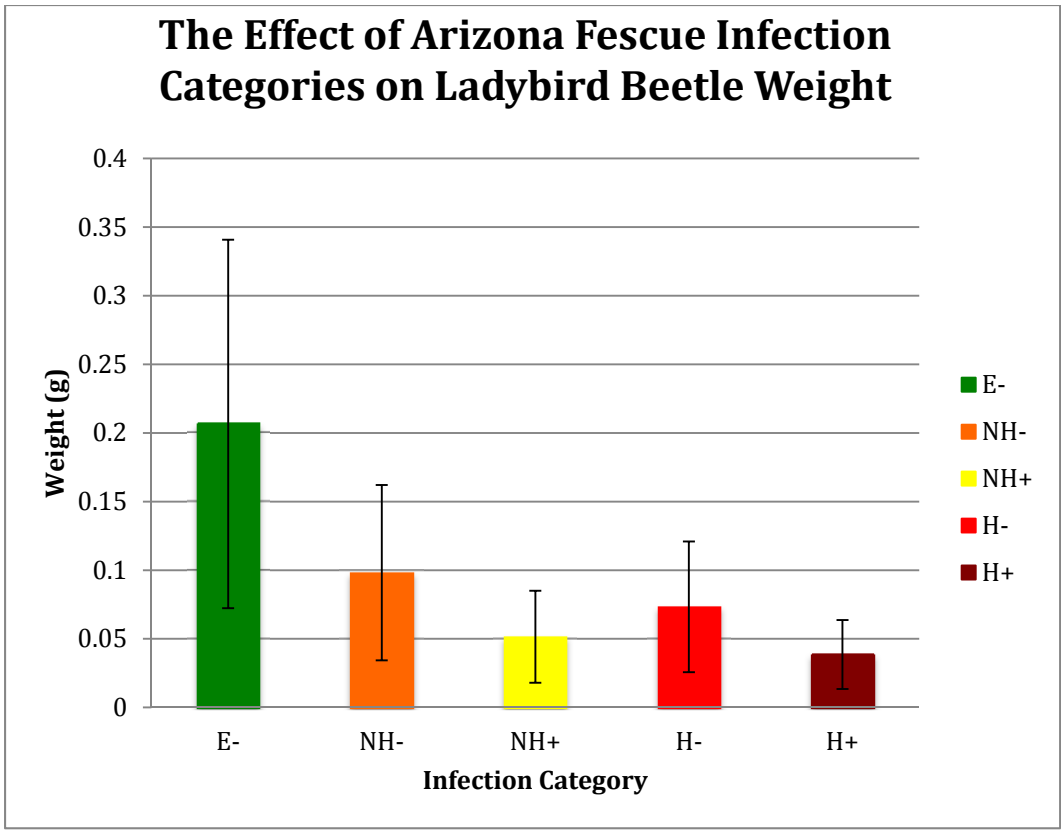

Figure 9. The Effect of Feeding on Aphids on Arizona Fescue with Different Infection Categories on Ladybird Beetle Weight. Box plots illustrate significant differences between E- vs. H+, however there were no other significant differences between mean weights of ladybird beetles. Tests were based on a confidence interval of 95%.

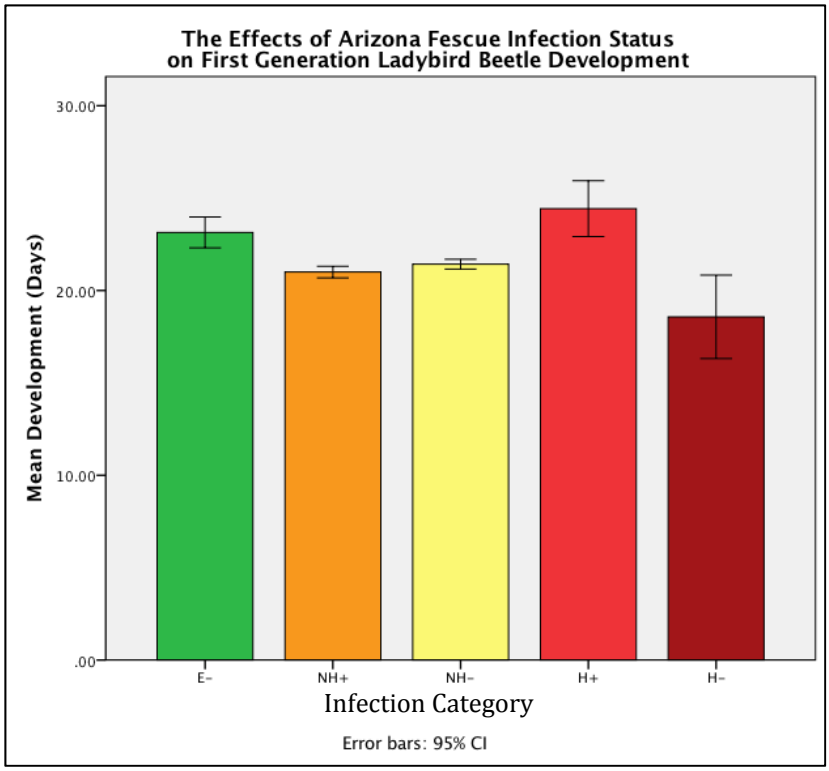

Figure 10. Effects of Feeding on Aphids from Arizona Fescue Infection Categories on Ladybird Beetle Developmental Time. All tests were based on a confidence interval of 95%

Ladybird Beetles Feeding Preference Experiment

Endophyte infection categories of the host plant with aphids affected ladybird beetle choice of plants overall ($p = 0.05$). Ladybird beetles were more likely to choose plants of all other infection categories (E-, H-, NH+) than to choose H+ plants (Tables 13). Furthermore, the odds of beetles choosing to feed on aphids on NH+ plants were 2.18 times higher than the odds of choosing NH- plants. The odds of choosing to feed on aphids on NH- plants were 2.03 times higher than the odds of choosing H- plants. Neither the number of aphids on the plants, the number of tillers, the height of the plant, nor the duration of a trial was significant predictors of ladybird

beetle preference for Arizona fescue of the five different endophyte infection categories. Again, odds represent the overall odds of choosing a particular status across all alternatives with which it was paired, and not the specific odds of choosing a particular status within a pair (Saari et al. 2013).

Table 13. Odds Ratios Comparing the Odds of Ladybird Beetle Preference. Ladybird beetle preference was for aphids feeding on infection category 1, over all pairs in which category 1 appeared, to the odds of choosing infection category 2, over all pairs in which category 2 appeared.

Category 1	Category 2	Odds Ratio (Category 1 / Category 2)	P-value
E-	H+	3.24	0.001
H-	H+	2.34	0.017
NH-	NH+	2.18	0.024
NH+	H+	2.17	0.028
NH-	H-	2.03	0.040
E-	NH+	1.49	0.212
NH-	E-	1.46	0.230
E-	H-	1.39	0.307

Effects of Grass Infection Status and Type on the Development of Second-Generation Ladybird Beetles

Infection categories of plants with aphids affected adult second generation ladybird beetle survival overall (between subject effect), F (4, 30) = 3.06, p = .03 (Table 14). The strength of the relationship between infection category and survival of adult second generation ladybird beetles, as assessed by η^2, accounted for 29% of the variance of the dependent variable (Table 14). Generally, survival was highest for beetles feeding on aphids from E- plants compared to NH+ plants, (p = 0.01), H+ plants, (p = 0.003), and H- (p = 0.02) plants (Table 15 and Figure 11).

The univariate GLM analysis of infection categories on second-generation ladybird beetle development was not significant, F (4, 30) = .377, p = 0.823 (Table 16). The strength of the relationship between infection category and ladybird development, as assessed by η^2, was not strong, η^2 = .048 (Table 16). There were no significant differences for the pairwise comparisons of infection status on ladybird beetle development (Table 17 and Figure 12).

Similarly, the GLM univariate analysis of the effect of infection categories on second-generation ladybird beetle mean weight was not significant, F (4, 30) = 1.31, p = 0.287 (Table 18). The strength of the relationship between infection status and ladybird beetle average weight, as assessed by η^2, was not strong, η^2 = .149 (Table 18). However, there were significant differences in the pairwise comparison for E- vs. NH+, p = .04 (Table 19 and Figure 13). Ladybird beetles that fed on aphids from E- plants had a significantly higher weight than those that were fed aphids from NH+ plants (Table 19).

Table 14. GLM Analysis of Arizona Fescue Infection Category Effects on Second-Generation Adult Ladybird Beetle Survival.

Tests of Between-Subjects Effects

Dependent Variable: Adultsurvived

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	83.029[a]	4	20.757	3.057	.032
Intercept	297.257	1	297.257	43.776	.000
Infection Category	83.029	4	20.757	3.057	.032
Error	203.714	30	6.790		
Total	584.000	35			
Corrected Total	286.743	34			

a. R Squared = .290 (Adjusted R Squared = .195)

Table 15. LSD Post-Hoc Test on Effects of Infection Categories on Second-Generation Adult Ladybird Beetle Survival.

Pairwise Comparisons

Dependent Variable: Adultsurvived

(I) Category	(I) Category	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
E-	NH+	3.714*	1.393	.012	.870	6.559
	NH-	2.429	1.393	.091	-.416	5.273
	H+	4.429*	1.393	.003	1.584	7.273
	H-	3.429*	1.393	.020	.584	6.273
NH+	E-	-3.714*	1.393	.012	-6.559	-.870
	NH-	-1.286	1.393	.363	-4.130	1.559
	H+	.714	1.393	.612	-2.130	3.559
	H-	-.286	1.393	.839	-3.130	2.559
NH-	E-	-2.429	1.393	.091	-5.273	.416
	NH+	1.286	1.393	.363	-1.559	4.130
	H+	2.000	1.393	.161	-.845	4.845
	H-	1.000	1.393	.478	-1.845	3.845
H+	E-	-4.429*	1.393	.003	-7.273	-1.584
	NH+	-.714	1.393	.612	-3.559	2.130
	NH-	-2.000	1.393	.161	-4.845	.845
	H-	-1.000	1.393	.478	-3.845	1.845
H-	E-	-3.429*	1.393	.020	-6.273	-.584
	NH+	.286	1.393	.839	-2.559	3.130
	NH-	-1.000	1.393	.478	-3.845	1.845
	H+	1.000	1.393	.478	-1.845	3.845

Based on estimated marginal means

*. The mean difference is significant at the

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

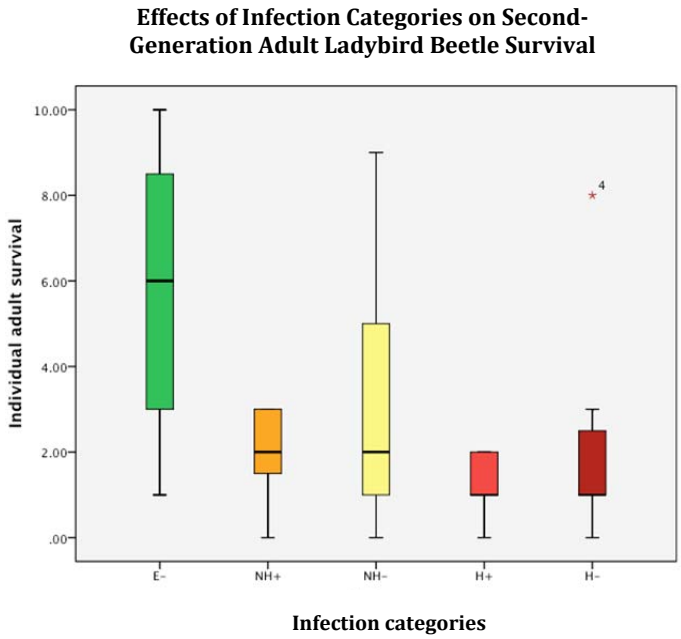

Figure 11. Effects of Infection Categories on Second-Generation Adult Ladybird Beetle Survival. Box plots illustrate means (± SD) of beetles that survived for the month of April. Outliers are marked with a star. All tests were based on a confidence interval of 95%.

Table 16. GLM Analysis of Arizona Fescue Infection Category Effects on Second-Generation Ladybird Beetle Development. The effect of infection status on ladybird beetle development was not significant, F (4, 30) = 0.377, p = 0.823

Tests of Between-Subjects Effects

Dependent Variable: Development

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	79.829[a]	4	19.957	.377	.823
Intercept	15603.457	1	15603.457	294.458	.000
Infection Category	79.829	4	19.957	.377	.823
Error	1589.714	30	52.990		
Total	17273.000	35			
Corrected Total	1669.543	34			

a. R Squared = .048 (Adjusted R Squared = -.079)

Table 17. LSD Post-Hoc Test on Effects of Infection Categories on Second-Generation Ladybird Beetle Development.

Pairwise Comparisons

Dependent Variable: Development

(I) Category	(I) Category	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
E-	NH+	-1.286	3.891	.743	-9.232	6.661
	NH-	2.571	3.891	.514	-5.375	10.518
	H+	.000	3.891	1.000	-7.947	7.947
	H-	2.429	3.891	.537	-5.518	10.375
NH+	E-	1.286	3.891	.743	-6.661	9.232
	NH-	3.857	3.891	.329	-4.089	11.804
	H+	1.286	3.891	.743	-6.661	9.232
	H-	3.714	3.891	.347	-4.232	11.661
NH-	E-	-2.571	3.891	.514	-10.518	5.375
	NH+	-3.857	3.891	.329	-11.804	4.089
	H+	-2.571	3.891	.514	-10.518	5.375
	H-	-.143	3.891	.971	-8.089	7.804
H+	E-	.000	3.891	1.000	-7.947	7.947
	NH+	-1.286	3.891	.743	-9.232	6.661
	NH-	2.571	3.891	.514	-5.375	10.518
	H-	2.429	3.891	.537	-5.518	10.375
H-	E-	-2.429	3.891	.537	-10.375	5.518
	NH+	-3.714	3.891	.347	-11.661	4.232
	NH-	.143	3.891	.971	-7.804	8.089
	H+	-2.429	3.891	.537	-10.375	5.518

Based on estimated marginal means

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

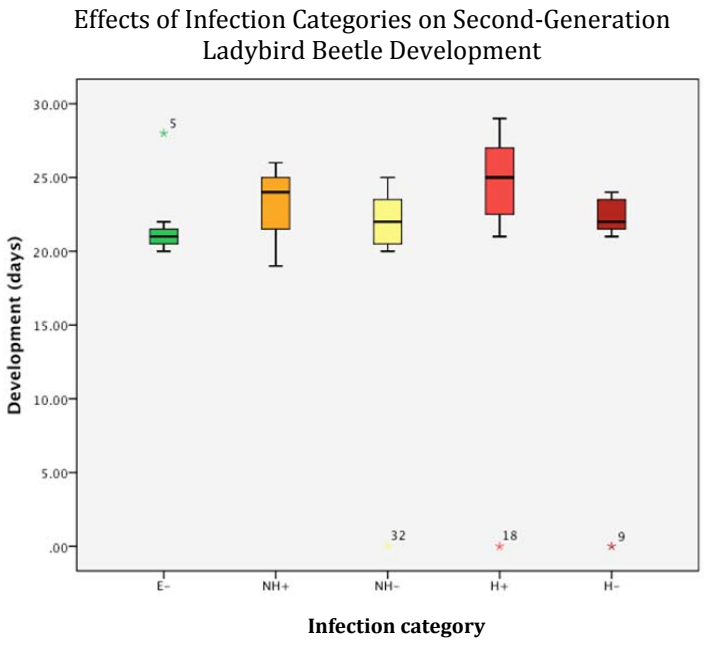

Figure 12. Effects of Infection Categories on Second-Generation Ladybird Beetle Development. Box plots illustrate means (± SD) of beetle development in days. Outliers are marked with a star. All tests were based on a confidence interval of 95%.

Table 18. GLM Analysis of Arizona Fescue Infection Category Effects on Ladybird Beetle Average Weight.

Tests of Between-Subjects Effects

Dependent Variable: Averageweight

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.000[a]	4	3.500E-006	1.314	.287
Intercept	.001	1	.001	198.295	.000
Category	1.400E-005	4	3.500E-006	1.314	.287
Error	7.991E-005	30	2.664E-006		
Total	.001	35			
Corrected Total	9.391E-005	34			

a. R Squared = .149 (Adjusted R Squared = .036)

Table 19. LSD Post-Hoc Test on Effects of Infection Categories on Ladybird Beetle Average Weight.

Pairwise Comparisons

Dependent Variable: Averageweight

(I) Category	(I) Category	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
E-	NH+	.002*	.001	.040	9.545E-005	.004
	NH-	.001	.001	.200	-.001	.003
	H+	.001	.001	.102	.000	.003
	H-	.001	.001	.127	.000	.003
NH+	E-	-.002*	.001	.040	-.004	.000
	NH-	-.001	.001	.408	-.003	.001
	H+	.000	.001	.645	-.002	.001
	H-	-.001	.001	.564	-.002	.001
NH-	E-	-.001	.001	.200	-.003	.001
	NH+	.001	.001	.408	-.001	.003
	H+	.000	.001	.710	-.001	.002
	H-	.000	.001	.799	-.002	.002
H+	E-	-.001	.001	.102	-.003	.000
	NH+	.000	.001	.645	-.001	.002
	NH-	.000	.001	.710	-.002	.001
	H-	.000	.001	.907	-.002	.002
H-	E-	-.001	.001	.127	-.003	.000
	NH+	.001	.001	.564	-.001	.002
	NH-	.000	.001	.799	-.002	.002
	H+	.000	.001	.907	-.002	.002

Based on estimated marginal means

*. The mean difference is significant at the

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

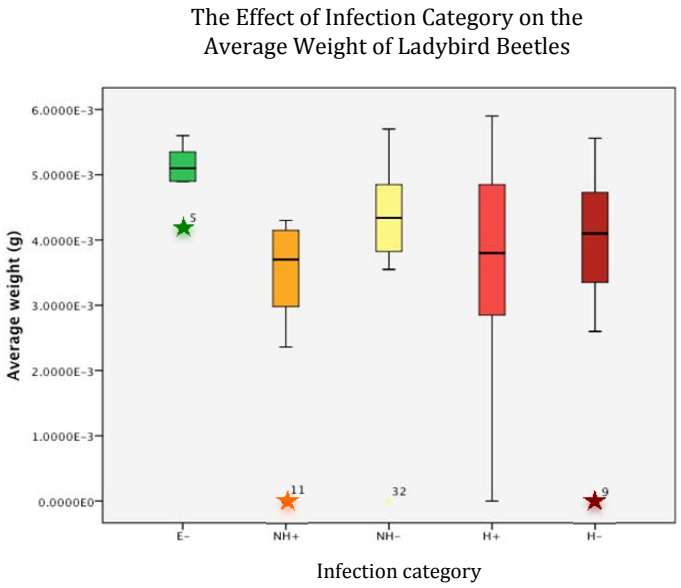

Figure 13. Box Plot Mean Values (± SD) of Ladybird Beetle Average Weight Per Infection Category. A star outside each of the boxes illustrates outliers. All tests were based on a confidence interval of 95%.

CHAPTER V

DISCUSSION

Asexual, vertically transmitted endophytes of cool season grasses were originally viewed as plant mutualists, primarily by providing defense against herbivores, seed predators and pathogens via the production of toxic fungal alkaloids (e.g., Clay 1990, Cheplick and Clay 1988). Although infected grasses have been shown to have strong deterrent and toxic properties against vertebrate and invertebrate herbivores, particularly in agronomic grasses, more recent evidence suggest the outcome of interactions is much more variable, and can range from antagonistic to mutualistic (e.g. Faeth 2002, Cheplick and Faeth 2009). This variability in the interactions of epichloid fungal endophyte is especially great in wild grasses, where outcomes of interactions of endophytes and their host grasses is now known to depend on plant and endophyte genotype and biotic and abiotic factors (e.g., Faeth and Cheplick 2009, Faeth et al., 1997, Saikkonen et al 1998, Faeth and Bultman 2002, Jani et al., 2010, Faeth and Saari 2012).

One biotic factor that can change the effect of alkaloids produced by symbiotic fungal endophytes in grasses is the presence of natural enemies. If alkaloids cascade up the food web and negatively affect predators or parasites of herbivores more than the herbivore themselves, then the defensive mutualism may be

thwarted or even reversed (Faeth and Saari 2012). Overall, my study showed that variation in endophyte genotype and associated endophytic alkaloids altered aphid abundances on both sleepygrass and Arizona fescue and altered aphids' feeding preferences. In Arizona fescue, the effects of genotype variation resulting from endophyte hybridization cascaded upward affecting feeding preference, development and survival of ladybird beetles.

Aphid Responses to Infection and Associated Alkaloids

In my study, I found that, as predicted by the defensive mutualism concept, that aphid herbivore abundances are reduced when feeding on sleepygrass plants with ergot alkaloids compared to those without alkaloids. Purportedly, NY clones of *R. padi* are more resistant to the alkaloids of infected grasses than other *R. padi* clones. (M. Rua and M. Dekkers, personal communication, Härri et al. 2008). Our experiments do not support this assumption, because NY aphids did not differ in abundances from NC aphids on sleepygrass, although there was a trend for NY aphids to show less negative response to ergot+ plants than NC aphids. Endophyte infected sleepygrass has been reported to contain varying concentrations of ergot alkaloids (ergonovine and lysergic acid amide), which are known to primarily deter vertebrates (Clay and Schardl 2002, Faeth et al., 2006, Jani et al., 2010).

However, my results also suggest a negative impact of these alkaloids on invertebrate herbivores given the reduction of aphid abundances. Alternatively, there could be additional unknown alkaloids produced by the endophyte in sleepygrass.

Recent evidence suggests that infected sleepygrass plants from the populations that I used in my experiment harbor genes for the ergot alkaloids ergonovine, lysergic acid amide, and chanoclavine, but also genes for indodipterpenes and perhaps peramine (Carolyn Young, personal communication). These other alkaloids, especially peramine (Leuchtmann et al. 2000) and indole dipterpenes (Popay and Hume 2011), may have negative effects on invertebrate herbivores.

For Arizona fescue, I also did not find strong difference in the NC and NY aphid strains. However, H+ plants had reduced aphid abundances compared to NH+, NH-, and H- plants. Furthermore, feeding preference matched performance results: aphids least preferred H+ plants relative to E- and NH- plants. Thus, my results support the hypothesis that hybridization in grass endophytes might lead to increased fitness of the host plant because of rapid influx of genes that confer increased herbivore resistance (Schardl and Craven 2003).

The mechanism for increased resistance and decreased preference by aphids is yet unclear. Infected Arizona fescue is known to produce peramine alkaloids (Faeth and Sullivan 2003) that are effective in deterring invertebrate herbivores (Leuchtmann et al. 2000, Clay and Schardl 2002,). Peramine is present in Arizona fescue in concentrations ranging from 3 – 12 ppm (Faeth and Sullivan 2003). The NH+ and H+ plants used in my study both produced peramine (E-, H- and NH- produce no peramine as expected) (Saari et al. 2013). However, the level of peramine did not vary statistically between NH+ and H+ plants ($t(18) = 0.55$, $p = 0.59$). So, at least peramine concentrations do not appear to explain reduced aphid abundances on

H+ plants. One explanation is that these hybrid endophytes of Arizona fescue possess genes for other alkaloids that were not measured in study. Recent evidence shows that both hybrid and nonhybrid endophytes in Arizona fescue also harbor genes for indole dipterpenes (C. Young, personal communication). It is yet unclear if either H+ or NH+ plants produce indolediterpenes (IDT) or if types and levels vary between these plants. My results with aphids would predict higher levels of IDT in H+ plants.

Another explanation is that there is some other physiological or morphological differences between the plant genotypes associated with H+ plants. Because NH and H endophytes may associate with certain plant genotypes (e.g., Saari et al. 2013), it may be that the plant genotype drives differences in aphid resistance. However, if this were true, one would expect differences in aphid abundances and preference between H- and NH- plants (plant genotypes with the effect of the endophyte removed). Because there were no differences, this suggests that the hybrid endophyte and its alteration to host phenotype is largely dictating differences in aphid resistance in Arizona fescue.

Endophytes and the Third Trophic Level – Ladybird Beetle Predators

Based only on aphid abundances and preferences, one might conclude that endophytes in native grasses act as defensive mutualists and protect the plant from at least aphid herbivores. Further, one might also conclude that hybridization acts to infuse genetic variation that additionally increases resistance of Arizona fescue to insect herbivores, supporting the predictions of Schardl and Craven (2003) and

Selosse et al. (2007). However, my experiments that include the third trophic level indicate this may not be the case.

At least in laboratory experiments, *Neotyphodium* endophytes cascade upward to affect the third trophic level, predators of the herbivores. I found that like the aphid herbivores, ladybird beetles avoid feeding on aphids reared on H+ plants relative to E-, H- and NH+ plants. There was no significant effect of infection or hybridization on ladybird size or length of development in the first generation, although there was a trend for smaller size and longer development on H+ plants relative to other categories. Choice by first generation adult beetles reflected growth and development trends: beetles avoided feeding on aphids on H+ plants relative to aphids on E-, H- and NH+ plants.

In the second generation, however, adult beetles reared on aphids from E- plants generally had higher survival than those feeding on aphids from other plant infection categories, and beetles feeding on aphids from H+ plants had the lowest survival overall (Figure 10). Generally, smaller size and longer development time in ladybird beetles results in lower fitness (Silva 2010). A stronger influence of endophyte infection and hybrid status on second-generation ladybird beetles than first generation beetles might be caused by time-delay effects, where that negative effects of endophytes might not be manifested until the later generations. Meister et al. (2006) suggested that endophytes might act slower by negatively influencing the life history of invertebrates through reduced longevity and fecundity. Therefore, it

appears that ladybird beetle predators avoid infected, and especially hybrid-infected, plants because growth and survival is lower when preying on aphids that are feeding on these grasses.

The mechanism of negative effects of endophytes on higher trophic levels is yet unclear. However, recent evidence shows that endophytic alkaloids can be detected in aphids feeding on infected grasses and in invertebrate predators feeding on the aphids (Fuchs et al. 2013 in press). Because there were no differences in peramine levels between H+ and NH+ plants, it is doubtful that differences in peramines explain these effects on ladybird beetles. As noted previous, genes for indolediterpenes have been recently discovered in H+ and NH+ Arizona fescue, but it is not yet known whether they differ in IDT levels or perhaps other alkaloids. However, like aphids, effects are clearly related to the hybrid endophyte rather than plant genotype, as there were no differences between H- and NH- plants on the third trophic level.

Including natural enemies alters conclusions based only upon aphid feeding and preference experiments. If the third trophic level is negatively affected by endophytes and the number of natural enemies decreased or predators avoids H+ plants, then an increase, rather than a decrease, in herbivore abundance on H+ plants might occur. (Jani *et al*., 2010; Faeth and Saari 2012). Thus, an indirect effect of bottom-up trophic cascades on natural enemies may have a negative effect on host plant fitness when natural enemies play a role in herbivore control (Dyer and Coley 2002, Saari and Faeth 2012) (Figure 3). If so, then including natural enemies may

invalidate both the defensive mutualism (Clay 1990, Clay 1988, Cheplick and Clay 1988) and the endophyte hybridization hypotheses. To more fully test these hypotheses, additional controlled experiments in the field with native grasses are needed.

Consideration of natural enemies may also explain a perplexing pattern in Arizona fescue. Hybrid endophytes are generally more frequent than non-hybrid endophyte among cool season grasses (about 2/3 are hybrids) and within grass species at the population level (Schardl and Craven 2003, Oberhofer et al. 2012). However in Arizona fescue, most infections are non-hybrid (55% NH+, 15% H+, 30%, E-, Saari et al 2013). Yet, H+ grasses grow, reproduce and compete better than NH+ grasses under some environmental conditions (Saari et al. 2012). H+ grasses may persist at lower frequencies in natural population because of the indirect and negative effects of endophytes on natural enemies. This hypothesis remains to be tested in controlled field experiments.

It is also possible that endophyte enhance the efficacy of natural enemies in natural populations. If natural enemies control herbivore populations (Fretwell, 1977; Oksanen et al., 1981), then they should have a positive effect on the host plant. Also, if endophytic alkaloids affect herbivores such that they are more susceptible to predation (e.g., slowing movement), but do not affect predators, then endophytes act in a positive feedback loop for the host grass by increasing top-down control (Härri et al., 2008). Again, additional experiments will be necessary to test this hypothesis.

Our experiments under laboratory conditions demonstrate that variation in endophyte genotype and hence, in alkaloids, can negatively affect herbivores, but may also have negative effects on natural enemies, which may nullify their benefits to the plant. However, in natural populations in the field, the outcomes of plant-endophyte-herbivore- natural enemy interactions can be complex (Figure 3). Because few wild grass-endophyte systems have been examined (Cheplick and Faeth 2009), generalizations about the nature of the endophyte-host interaction are still premature. Future studies that examine multitrophic interactions that consider genetic variation of plant and endophyte and abiotic and biotic environmental factors that affect interaction outcomes are needed (e.g., Cheplick and Faeth 2009, Faeth and Saari 2012).

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